Exosome-mediated Differentiation of Adipose-derived Mesenchymal Stem Cells to Trabecular Meshwork Cells

A Thesis From

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Abstract

Glaucoma is the second leading cause of blindness and affects over 70 million people worldwide. Intraocular pressure (IOP), a well-established risk factor for primary open angle glaucoma, the most common form of glaucoma, is primarily regulated by aqueous humor outflow through drainage tissues, specifically the trabecular meshwork (TM). In primary open angle glaucoma, TM cellularity is significantly decreased in comparison to age-matched healthy eyes, presumably limiting the TM's ability to regulate IOP. It is hypothesized that regenerating TM cellularity in a glaucomatous eye would lead to proper IOP and aqueous outflow regulation, preventing further vision loss associated with glaucoma. Towards this, we investigated the use of TM cell-derived exosomes, small secreted particles 40nm-1μm in diameter, to induce differentiation in adipose-derived mesenchymal stem cells (MSC) toward a TM lineage. Using established TM characterization and exosome isolation methods, we co-cultured TM-derived exosomes and MSCs, along with control TM and MSC cells, for two weeks and observed changes relative to untreated MSCs and TM cells. While experimental groups were not significantly different from untreated MSC cells, trends relating exosome concentration and TM phenotype were observed. Importantly, untreated TM cells were found to be significantly different from past research in three out of four characterization assays, indicating phenotypic loss during cell culture without exosome treatment. These results highlight the importance of culture conditions necessary to attain and maintain the TM phenotype. Further, trends from this study support the idea that exosomes play a role in TM differentiation and inform efforts to develop glaucomatous, stem cell-based regenerative therapeutics.

Introduction

Glaucoma, the second leading cause of blindness, affects over 70 million people worldwide, and its incidence is expected to continue to rise with an aging population[1]. Intraocular pressure (IOP), a well-established risk factor for glaucoma, is primarily regulated by aqueous humor outflow through the drainage tissues in the anterior eye, specifically the trabecular meshwork (TM)[2]. In primary open angle glaucoma, TM cellularity is significantly decreased in comparison to age-matched healthy eyes, presumably limiting the TM's ability to regulate IOP[3]. It is hypothesized that regenerating TM cellularity in a glaucoma-affected eye would lead to proper IOP and outflow regulation.

Stem cells are undifferentiated cells capable of differentiating into other cell types. Stem cells have become more and more popular for regenerative medicine, since their first use in hematopoietic stem cell transplants for treating radiation damage[4]. In 1998, human stem cells were derived from embryos, contributing both scientific understanding of how human tissues form and regenerate[5] but raising ethical questions about their use, as the embryo was destroyed during the procedure. To avoid these ethical complications, Takahashi *et al.* developed a process to create induced pluripotent stem cells (iPSCs), adult cells reprogrammed to an embryonic-like stem cell state[6]. While iPSCs can avoid immune rejection and ethical hurdles, they still pose risk of teratoma tumor formation and require costly, lengthy reprogramming methods which are difficult to translate clinically. Alternatively, adipose-derived stem cells have been proposed as an effective source of tissue regeneration, capable of differentiating into multiple cell types while being widely available from liposuction procedures[7]. Recently, we have been able to characterize differences between adipose-derived mesenchymal stem cells (MSCs) and TM cells as a first step towards using adipose-derived stem cells for TM regeneration[8]. Furthermore, preliminary results show evidence that MSCs can differentiate through co-culture with TM cells; however, only a low efficiency, inconsistent

degree of differentiation was reached[9]. Interestingly, stem cells treated with TM-derived conditioned media seemed to express further and more consistent differentiation, further justifying this study. For these reasons, we investigated effects of adding endogenous agents to the culture medium to more consistently induce MSCs to differentiate into phenotypical and functional TM cells. Exosomes—cell-borne membrane-bound particles ranging from 40 to 1 μm[10]—represent a potentially effective approach for stem cell differentiation since they are inexpensive, readily available, and simple to use. Here, we explore their application for TM differentiation.

Literature Review

Glaucoma and Trabecular Meshwork

Currently, medical and surgical glaucoma treatments focus on decreasing intraocular pressure (IOP) as their primary goal[11]. IOP is related to flow of aqueous humor (AH), a clear fluid produced by the ciliary processes to provide nutrients to avascular tissues in the anterior chamber. Importantly, elevated IOP is a well-established risk factor for glaucoma, and production rate of AH remains approximately constant throughout glaucoma progression[12-14]. Therefore, elevated IOP is primarily a result of poor AH outflow, regulated by the drainage tissues of the anterior chamber, specifically the trabecular meshwork (TM)[2]. The TM regulates IOP by modulating AH outflow to the circulatory system[15], and actively 'cleans' debris from exiting AH through phagocytosis[16]. In 1981, a study of 35 subjects, ranging in age from birth to 81 years of age, found that in primary open angle glaucoma, TM cellularity is significantly decreased in comparison to age-matched healthy eyes[3]. Decreased cellularity is theorized to inhibit the TM's ability to regulate IOP, and given this reduced TM cellularity and strong connection to IOP regulation, it is believed that recellularizing the TM in a glaucoma-affected eye would lead to proper IOP and outflow regulation, and reduction in glaucoma-associated symptoms.

Paracrine Signaling and Exosomes

Paracrine signaling is one of many categorizations of intercellular communication. Unlike endocrine signaling, the long-distance and slow communication between tissues and cells of the body, paracrine signaling takes place between close or adjacent cells. A proposed major vehicle for paracrine signaling is exosomes: Exosomes are small (40-100nm) collections of proteins and other source-cell dependent material encased in a lipid bilayer membrane, released either when multivesicular bodies combine with plasma membrane or by pinching off directly from the plasma membrane[17]. Exosomes were first observed in 1983 when Pan et al. and Harding et al. described small (~50nm) vesicles ejected from blood reticulocytes[18, 19], although the term 'exosomes' did not appear until a few years later when Rose Johnstone coined the term[20]. The mRNA and microRNA cargo of exosomes was first analyzed in 2007, revealing not only differences in mRNA and microRNA from those found in cells, but also that the mRNA is fully functional[21]. Exosomes are secreted from most, if not all, cell types, and have been implicated in bodily processes such as immune suppression, inflammation, genetic information encoding, waste management, and coagulation[22].

Regenerative Medicine

Exosomes are understood to play a significant role in stem cell differentiation[22]. One study investigated the possibilities of exosome-induced differentiation for bone regeneration, stating the results "show the potential of cell derived exosomes in bone regenerative medicine and opens up new avenues for future research[23]." Furthermore, researchers have also used exosomes as paracrine signals to differentiate stem cells into cardiomyocytes, with treated cells beginning to beat with cardiac rhythm[24].

Focusing on regenerating the trabecular meshwork, several studies have shown potential for stem cell tissue regeneration therapies. In a very recent study, induced pluripotent stem cells (iPSCs) differentiated to a TM lineage were delivered into the TM *in vivo* in mice. Over a 9 week period, the

treatment restored phenotypical TM function, regulating IOP and preventing retinal ganglion cell axonal loss[25]. While promising, iPSCs for TM regeneration are challenging from a clinical translation standpoint due to complex reprogramming and tumorigenic potential. Alternatively, multipotent stem cells isolated from the TM tissue have also been differentiated to TM cells through application of aqueous humor, though the differentiated cells were functionally characterized by only their phagocytic properties[26]. Adipose-derived stem cells have been reviewed and proposed as an effective source of tissue regeneration, capable of differentiating into multiple cell types while being widely available from liposuction procedures[7]. Recently, we have characterized differences between adipose-derived mesenchymal stem cells (MSCs) and TM cells, specifically examining differences in myocilin expression after exposure to dexamethasone, cell markers, contractile properties, and phagocytic behavior[8]. TM cells have been shown by Stamer *et al*. to secrete exosomes with a protein profile containing common exosome markers like CD-9 and CD-63, as well as TM-specific proteins like emilin-1 and myocilin[27]. Given the potential for exosome-driven differentiation towards a TM lineage and the accessibility of the exosomes themselves, a method for differentiating widely available MSCs into TM cells using only exosome-mediated signaling would represent an more economical and effective treatment option for regenerating the TM cellularity of glaucoma-affected eyes.

This project investigated the application of exosomes from TM cells as a mechanism to achieve MSC to TM lineage differentiation, expanding knowledge about exosomes, the TM, and glaucoma treatment options.

Methods and Materials

Cell Sourcing and Culture

Human TM cells were isolated and characterized by researchers at Duke University (acknowledgments to Dr. Daniel Stamer). For collecting conditioned media, TM cells were cultured and passaged in T-75 flasks (Falcon) in 1% exosome-free FBS (SystemBio), 1g/L glucose Alpha MEM medium (EFM). TM cells were allowed to adhere overnight and media was replaced every 2-3 days with fresh nano-clean EFM until cells were prepared for experiment.. Adipose-derived mesenchymal stem cells (Lonza), were characterized and cultured in T-75 flasks (Falcon) in 20% FBS (Corning), 4.5g/L glucose Alpha MEM medium for 1 day, then washed and replenished with 20% FBS, 4.5g/L glucose media every 5 days until cells were prepared for experiment.

Exosome Isolation and Characterization

After culturing for two to three days, conditioned media from hTM cells was collected and transported on ice to undergo ultracentrifuge exosome isolation, following established methods[28]. Briefly, conditioned media was centrifuged at 2,000*g* (Sorval ST8R Centrifuge, ThermoFisher Scientific) for 20 minutes to remove cells. The supernatant was then centrifuged at 10,000*g* for 30 minutes to remove cell debris. The supernatant was then transferred to polycarbonate aluminum ultracentrifuge bottles (Beckman Coulter) and centrifuged at 100,000*g* for 70 minutes with the Allegra X-15R Ultracentrifuge (Beckman Coulter). The supernatant was drawn, leaving 1mL at bottom containing the invisible exosome pellet. exosome pellet was then resuspended in PBS and centrifuged at 100,000*g* for 70 minutes again. Supernatant was aspirated, and exosome pellet was collected and resuspended in PBS and a protease inhibitor cocktail (Thermo-Scientific #78430) at -80ºC until analysis or application to stem cell cultures. Fresh reagents were used throughout.

Size and concentration of particles in the isolated pellets were measured with nanoparticle tracking analysis (NTA) using ZetaView PMX 110 (Particle Metrix) and ZetaView Version 8.02.28 (acknowledgments to Yutao Liu at the University of Augusta) Isolated pellet samples were diluted with PBS (Life Technologies) per manufacturer's specifications prior to NTA. After calibrating with 100nm polystyrene particles, NTA measurements were recorded and analyzed at 11 positions with the ZetaView system, maintaining a temperature between 22℃ and 23℃. Zeta potential was measured using 0.05X PBS to adjust conductivity to near 500 μS/cm[29].

Differentiation Experiments

Using established exosome isolation methods, conditioned media from cultured TM cells was separated into exosome isolates and leftover decanted media, including all the constituents of conditioned media except exosomes. Here, we called the leftover media conditioned media supernatant (CMS). At 90% confluency, MSCs were passaged to 24 well plates at 5,000 cells/cm^2. MSCs were split into six groups and treated with corresponding media: Only EFM; 1X concentration exosomes with CMS; CMS only; and 1X, 2X, and 4X exosome concentration in PBS (See Table 1). TM cells, upon 90% confluency, were also passaged to 24 well plates at 5,000 cells/cm^2 and treated with EFM only. Media was replaced every three to five days. Cells were treated for 2 weeks, followed by differentiation assessment.

Differentiation Assays

We have previously determined assays suitable for detecting differentiation from MSCs to TM cells[8]. Briefly, differentiation was evaluated through four assays:

Dexamethasone Induction of Myocilin

TM cells express myocilin in response to treatment with dexamethasone (DEX)[30]. Cells were cultured on 1% gelatin-coated glass coverslips in 24-well plate wells and treated with culture media for 7 days, replacing media every three days. Cells were probed with 200 μg/mL MYOC goat antibody, then

secondary donkey anti-goat Alexa Fluor 647, stained with DAPI and then imaged using a Leica DM^ Fluorescent Microscope with the 10X objective. Images of 3 replicate dexamethasone-induced samples and non-induced samples were analyzed for mean fluorescence intensity of myocilin and nuclei count (DAPI) using ImageJ. Ratios of myocilin fluorescent intensity and cell count were averaged for each dexamethasone treated experiment and compared to respective untreated controls

Collagen Gel Contractility

Calf skin Collagen I (MP Biomedical) was prepared (under sterile conditions) in 0.2% (v/v) acetic acid at a concentration of 6 mg/mL. The solution was gently agitated at 4oC for 1 to 2 days to ensure the collagen was completely dissolved. The collagen solution was then diluted to 3 mg/ml (0.1% acetic acid) with sterile H2O.

Cells were counted, centrifuged, and resuspended in serum-free media at 900,000 cells/mL. Immediately prior to plating, the 3mg/ml collagen was neutralized with NaOH to a pH of 7.4, matching that of DMEM media. Cells in serum-free medium were resuspended in the neutralized collagen solution at a 2:1 cell/collagen ratio (600, 000 cells/ml) and plated in 96 well plates, 80 µL per well. Cultures were incubated 20 minutes at room temperature to allow the collagen to solidify into gels. 80 µL of serum-free media was added to each well and cultures were maintained in a humidified atmosphere, 37°C, 5% CO_2. 24 hours after seeding, a sterile pipette tip was used to release each gel from the walls of its well. Gels were imaged (ChemiDoc MP, Bio-Rad) 12 hours later. Images of any gels that did not release from the wells were discarded and not considered, as adherence forces interfered with contractile forces. Images obtained of the surface area of contracted gels and 96-well plate were compared and analyzed using ImageJ analysis software.

Phagocytosis Assay

Cells were seeded at 50,000 cells/cm^2 onto 48 well plates in Hanks' balanced salt solution (HBSS) supplemented with 5% FBS and 2mM L-glutamine[31]. After 24 hours, pHrodo green e.coli BioParticles

(Life Technologies) were reconstituted in HBSS media as per the manufacturer's protocol and added to cells. After a 4-hour incubation at 37ºC, cells were detached with 0.05% Trypsin/EDTA and cells were processed by flow cytometry for particle fluorescent intensity (100 µL/min Attune NxT, Thermo-Scientific).

Reverse Transcription Polymerase Chain Reaction Analysis

RNA was isolated from samples using TRIzol reagent (Life Technologies) and further purified using Aurum Total RNA Mini Kit (Bio-Rad), per the manufacturer's protocol. RNA concentration and purity were determined using a Nanodrop 2000C spectrophotometer (Thermo-Scientific). Reverse transcription to clonal DNA (cDNA) was performed on 1µg RNA samples per the manufacturer's protocol (Bio-Rad). cDNA samples were analyzed against seven primer pairs (Supplementary Table 1) (Du et al., 2012, Spandidos et al., 2008, Spandidos et al., 2010, Wang and Seed, 2003, Ye et al., 2012). SYBR green reagent (Bio-Rad) was used to quantify DNA amplification in real time throughout the PCR cycle (StepOne Plus Real Time PCR System, Applied Biosystems). The resulting cyclic threshold (C_T) values were normalized to the geometric mean of three reference genes (GUSB, YWHAZ, and TATA BP; see Table 2) and further normalized against untreated MSC samples using the $2^{-\Delta\Delta C_T}$ methodology (Livak and Schmittgen, 2001).

Results

Exosome Isolation and Analysis

Exosome isolates from conditioned media, ranging from 60µL to 80µL, were analyzed for particles between 7 and 615 nm by NTA (n = 10). Median particle diameter ranged from 113 and 140 nm (Figure 1). Particle concentrations ranged from 4.4 to 29.6 million particles per cm^3, with a mean of 18.5 million

particles per cm^3 (Figure 2). These median particle diameter and concentration fall within reasonable ranges for that of exosomes.

Differentiation Assays

Dexamethasone Induction of Myocilin

Cell nuclei were stained with DAPI and myocilin was immunolabeled. Mean myocilin fluorescence varied across experimental groups with dexamethasone treatment. Average fluorescence among TM, 2X, and 1X groups more than quadrupled that of MSC, CMS, ExoCMS, and 4X. After 1 week dexamethasone treatment, no groups changed significantly except for CMS, increasing by about 160%. However, this percentile increase may be explained by its already-low fluorescence for both dexamethasone-induction and untreated groups (Figure 3). Example images of TM, 4X, and MSC sample groups can also be found in Image 1.

Contractility

Pictures taken 12 hours after gel release were analyzed. Experimental gels which failed to detach from the 96-well walls were not included in analysis, as adhesion forces on the wall interfere with the contractile forces and cannot be compared to wells where full detachment occurred. Area, as a percentage of the total well area, can be visualized in Figure 4. No sample group, including TM controls, could be proven significantly different in area from MSC controls, on average: See Table 2 for % well area and p-values. However, trends suggest changes in area that correlate with exosome concentration: lessening contractility and more TM-characteristic contractile behavior with higher exosome concentration. Results also allude to a synergistic effect of exosomes and EFM, which combined to achieve a stronger effect.

Phagocytosis

For assessing phagocytosis, cell samples were incubated with fluorescent phagocytic particles for 4 hours before fluorescent intensity was measured by flow cytometry. Fluorescence in samples not given

phagocytosis particles consistently held between 950 and 1060 (arbitrary units, AU) in value. Samples given phagocytic particles ranged from 7500 to 29000 (AU) in value.

Untreated MSCs expressed the highest fluorescent intensity, with 28,773 (AU). Sample groups were not significantly different from each other, as a result of high variability within each sample group. However, averaged median fluorescence among each experimental group rested below that of TM control samples, which were much lower than that of the MSC control samples. Median fluorescence trended towards TM behavior across all experimental groups, though again not statistically significant. Fluorescence values in sample groups given particles can be visualized in Figure 5.

RT-PCR Protein Quantification

To compare and quantify experimental groups, transcript expression C_T results for the five differentiation-associated genes (tPA, Mucin 1, CHI3L1, MGP, and MYOC) were first normalized to the geometric mean of three reference genes, generating Δ^{C_T} values. These Δ^{C_T} values were then compared to all other groups, including that of the MSC controls for each gene. To avoid cross-comparing different plates, normalization calculations were derived from and applied to wells of the same plate. The MSC sample's Muc1 expression immediately reached threshold, indicating a possible error in analysis. Each sample's gene expression can be viewed in Table 3, and visualized in Figure 6. Through Tukey's Multiple Comparisons Test, no significant differences could be established between any sample groups in all genes except myocilin. Within Myocilin, a few groups differed significantly: TM and 1X groups ($p=0.0068$), MSC and 1X groups ($p=0.0079$), CMS and 1X groups ($p=0.0219$), ExoCMS and 1X groups ($p=0.0067$), 4X and 1X groups ($p=0.0073$), and 2X and 1X groups ($p=0.0091$). From this, 1X concentration exosome group shows a significant departure from all the other groups, though it departs from both TM and MSC control groups.

Discussion

Exosomes may represent a novel approach for understanding the development of trabecular meshwork cells and restoring lost trabecular meshwork functionality in primary open angle glaucoma. Utilizing established characterization and exosome isolation methods, we explored functional and characteristic changes in MSC's treated with exosomes with a goal of understanding the role of exosomes in developing stem cells towards a TM lineage. If differentiation towards a TM lineage with MSC's using exosomes can be shown possible, exosomes may play a large role in accelerating TM regeneration therapies and better understanding embryological development of TM tissue.

First, we established that exosomes can be isolated from TM cells (See Figure 1 and 2) and these particles' median diameter was similar to exosomes, around 130nm. There is no evidence the exosomes were improperly manipulated between isolation steps and differentiation experiments, but potential evidence was not necessarily observable, because analysis of media at transfer steps would destroy the sample. There is also no evidence that freezing the exosomes for storage were damaging the cells, as exosomes were analyzed after freezing, and freezing at -80º C is routinely performed for exosome storage.

Control Groups Analysis

Interestingly, in many experiments the TM samples were not significantly different from MSC controls. This can be explained by a few general conditions. The first possibility is these experiments or tests are not accurately tracking differences between TM cells and MSCs. This seems unlikely, however, given the body of work demonstrating clear and significant differences that formed the backbone of this project[8, 32, 33]. Second, it's possible the experiments themselves may not have been executed correctly, resulting in null results. It's not entirely clear that this is the case, though, as the samples were otherwise without disqualifying problems, and most are common protocols run by investigators many times

correctly. A third possibility is the TM and MSC cells used were unhealthy or otherwise abnormal, compared to average or typical TM and MSC cells or at the very least, compared to those in research characterizing differences between MSC and TM cells. To investigate, we obtained datasets from previous characterization analysis[32, 34] and compared results from each TM and MSC pair. Some methods used in past characterization work differed slightly from methods in this work, though we believe a comparison is both possible and reasonable.

Dexamethasone Induction of Myocilin

Methods for gathering dexamethasone expression differed from characterization work: Past characterization work utilized western blotting and fluorescent flow cytometry, while we used fluorescent image analysis to retain cell counts. To analyze, we will be using only fluorescent flow cytometry, because both fluorescent flow cytometry and image analysis measured antibody fluorescence per cell, which can be compared. Average median fluorescence of each group was compared with its' groups twin: MSC groups could not be shown to be significantly different ($p=0.3826$). TM groups, however, were significantly different with p-value of 0.0462 (Figure 7).

Contractility Assay

Contractility assay methods were retained from previous characterization work. However, contractility, as a percentage of area filled by cell gels, was not directly documented at 12 hours in past research. To provide a 12-hour data point, we extracted a linear regression using log of hours from past research data points and extrapolated a $t=12$ hours well area with 95% confidence intervals for analysis (Figure 8). The $t=12$ hours interpolated points were compared with our data (Figure 9). With Sidak's Multiple comparisons test, we observed significant differences in the TM groups ($p<0.0001$), but no significant difference in the two MSC groups ($p=0.7502$).

Phagocytosis

Methods for phagocytosis assay were retained from previous characterization work, though variance among experiments should always be considered. Average fluorescence was compared using a student's T-test. MSC groups for past work and ours was not shown to be significantly different: 25,801.0 against 24,898.2 with a p-value of $p= 0.077$. However, TM group's phagocytic activity differed significantly: 13,869.0 against 42,375.8 with a p-value of $p< 0.0001$ (Figure 10).

RT-PCR Protein Quantification

RT-PCR protocol was similar to previous characterization results. Genetic expression of each gene after normalizing to housekeeping was compared with student's t test. Mean expression of any genes between the two MSC and two TM groups could not be shown significantly different from each other: p-values were as high as 0.99 (Figure 11).

As we compare this study's TM and MSC groups to past research, we see no strong differences between the MSC groups, but TM groups differ significantly, across three of the four characterization assays and accounting for intra-experimental variance. Our TM control samples were not similar to those in past characterization research[8]. Though our TM and MSC cells were characterized by their sources, including multiple cell lines would have helped clarify any sourcing or cell line abnormalities, and we hope to include multiple cell lines in future research.

Under the assumptions of a typical cell line and source, the experiments having sufficient power in distinguishing the two cell types, and the experiments been run correctly, a change must have occurred in the TM cells away from normality during the experiment. Characteristic cells grown in cultures dissimilar to their natural conditions can lose characteristics[35, 36]. Important physiological and environmental factors not present *in vivo*, but likely present here, include nutrient-rich media, two-dimensional culture, high passage number, and hard culture flask surface. The TM, neighboring

Schlemm's canal at the limbus or cornea-sclera margin, is avascular which of course differs from cell culture media in nutrient and oxygen content.

Experimental Groups

No experimental group showed consistent statistical differences from MSC samples in most characterization assays. That's not to say nothing can be said: Trends in many assays suggest samples given exosomes tracked similar results as this work's TM cells, including Phagocytosis, Contractility, and especially RT-PCR. With the presence of exosomes, MSCs seem to trend towards TM characteristics. We believe steps could be taken in future research to improve the power of these experiments, including: Larger cell sample sizes, as many cells were removed during steps in characterization assays, and more powerful statistical analysis methods for highly variable results like RT-PCR data.

Conclusion

If TM control groups in this study lost their characteristic traits in culture, these results cannot inform the possibilities of exosomes for MSC differentiation to TM phenotype. Though development of MSC-to-TM differentiation through application of exosomes and its implications for novel treatment options and understanding remain a possibility, this study presents a circumstance in TM research that threatens past and future research. Namely, culturing TM cell lines in culture similar to that of this study may encourage changes in TM cells away from a recognizably TM lineage. We propose further research to investigate characteristic changes in TM as a result of common culturing practices, to both justify research using cultured TM cells and better learn about TM behavior and response in varying environments.

Tables and Figures

	Group Name	Description
1	MSC Control or “MSC”	EFM only
	TM Control or “TM”	EFM only
2	1X Concentration exosomes with CMS or “ExoCMS”	1X Concentration exosomes added to CMS
3	4X Concentration exosomes or “4X”	EFM and 4 Million Particles/mL added to EFM
4	2X Concentration exosomes or “2X”	EFM and 2 Million Particles/mL added to EFM
5	1X Concentration exosomes or “1X”	EFM and 1 Million Particles/mL added to EFM
6	Conditioned Media Supernatant or “CMS”	Exosome-depleted conditioned media only

Table 1: Sample group names and description of treatment media. Two controls, representing both cell types, received only fresh media. The others, received CMS, exosomes, or a combination of both.

Primer Name		Category	Primer Sequence (5' to 3')	Reference
Chitinase 3-Like 1 (Chi3L1)	Forward	TM Cell Marker	CCTTGACCGCTTCCTCTGTA	[26]
	Reverse		GTGTTGAGCATGCCGTAGAG	
Matrix GLA Protein (MGP)	Forward		GCCGCCTTAGCGGTAGTAAC	[26]
	Reverse		TCTCTGCTGAGGGGATATGA	
Tissue Plasminogen Activator (tPA)	Forward		AGCGAGCCAAGGTGTTTCAA	PrimerBank 132626665c1[26]
	Reverse		CTTCCCAGCAAATCCTTCGGG	
Myocilin (MYOC)	Forward		AAGCCCACCTACCCCTACAC	[26]
	Reverse		TCCAGTGGCCTAGGCAGTAT	
Mucin 1 (MUC 1)	Forward	MSC Marker	CCATTCCACTCCACTCAGGT	[26]
	Reverse		CCACATGAGCTTCCACACAC	
TATA Binding Protein	Forward	Reference Marker	CCACTCACAGACTCTCACAAC	PrimerBank 285026518c1[37, 38]
	Reverse		CTGCGGTACAATCCCAGAACT	
Tyrosine 3/tryptophan 5-monoxygenase activation protein (YWHAZ)	Forward		CCTGCTGAAGTCTGTAACTGAG	PrimerBank 208973243c1[26]
	Reverse		GACCTACGGGCTCCTACAACA	
Beta-Glucuronidase (GUSB)	Forward		GTCTGCGGCATTTTGTCGG	Primer-BLAST [36]
	Reverse		CACACGATGGCATAGGAATGG	

Table 2: Primer sequences for RT-PCR gene expression assay. References indicate motivation for each genes' use. All were used in the work by Snider et al, as TM characterization markers or housekeeping genes[8].

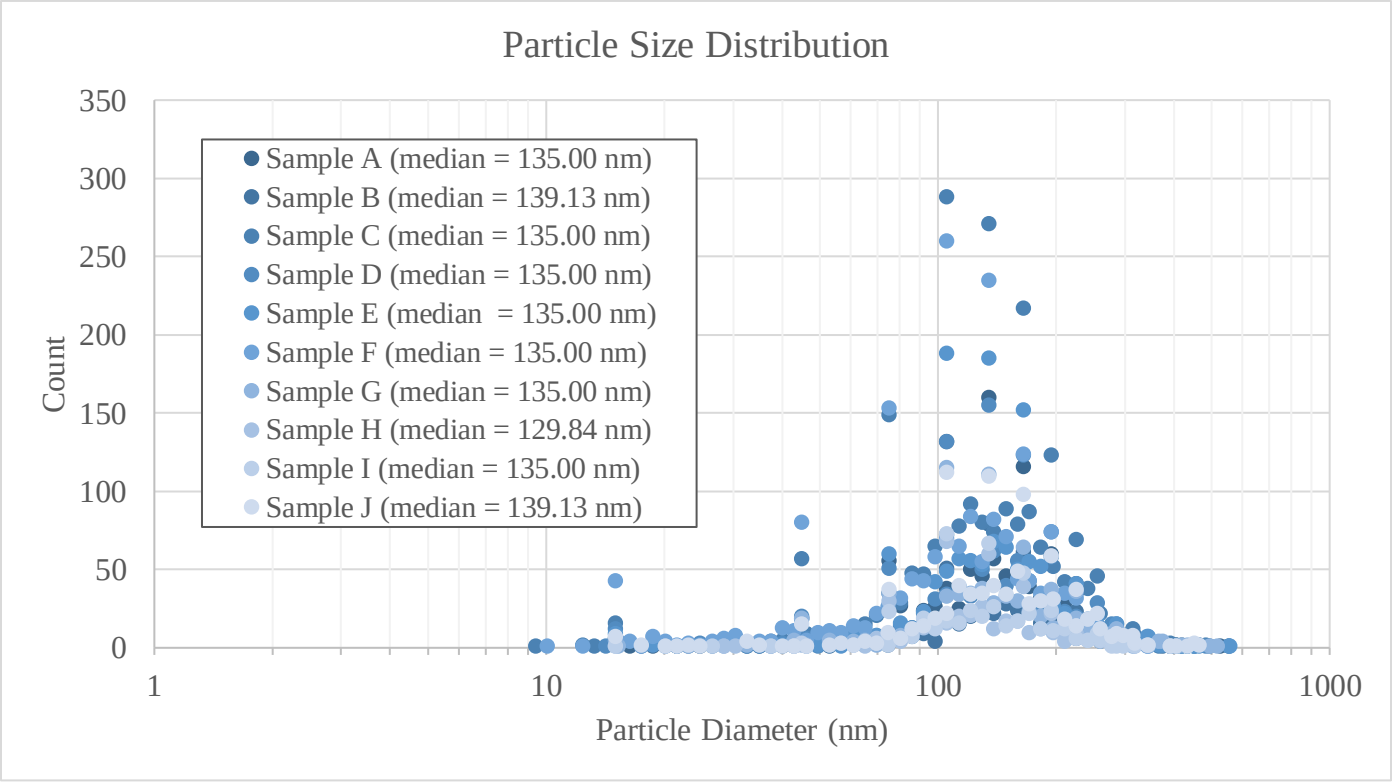

Figure 1: Histogram plot of particle size distribution from ten 80µL isolates from TM cells after ultracentrifugation steps. Median particle diameter consistently rests at 135nm, similar to most EVs.

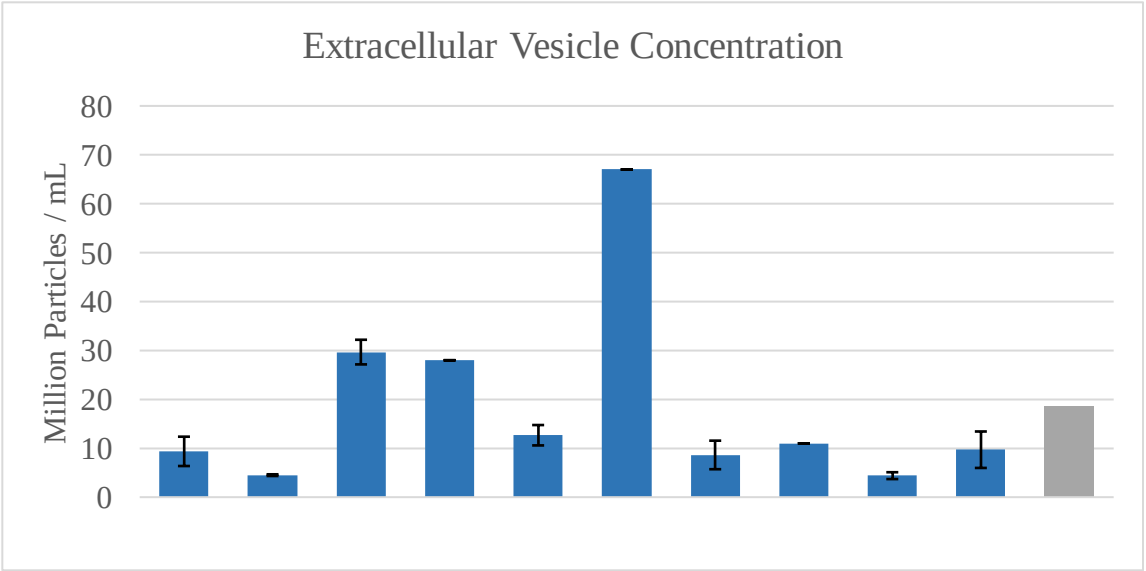

Figure 2: Mean particle concentration from ten 80µL TM cell sample isolates, in million particles per mL. Error bars signify standard deviation of variance. Average concentration of all samples shown in grey.

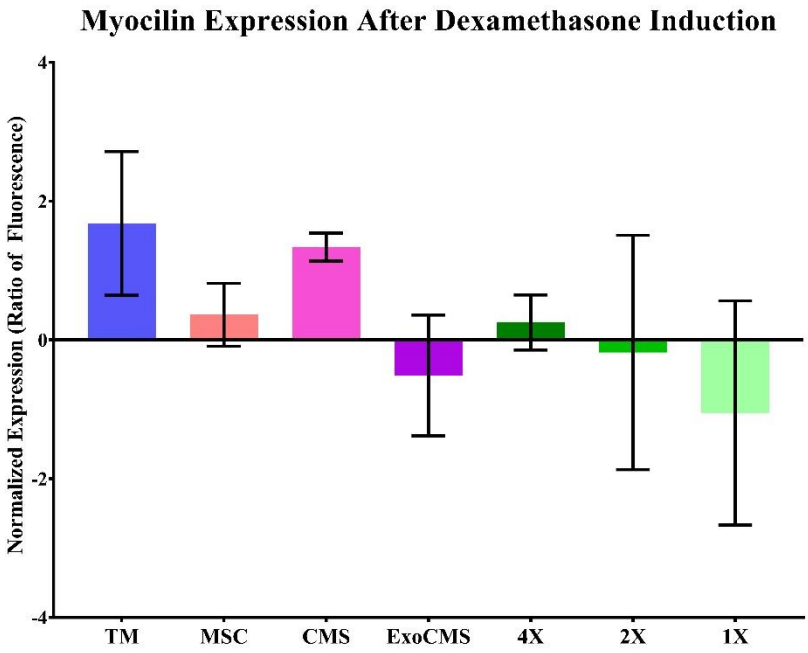

Figure 3: Myocilin expression as represented by antibody fluorescent stain after treating with dexamethasone for a week. Error bars represent standard deviation. Fluorescence values here are normalized from baseline fluoresence taken from cells not given dexamethasone. ExoCMS and 1X groups expressed less myocilin when exposed to dexamethasone for a week.

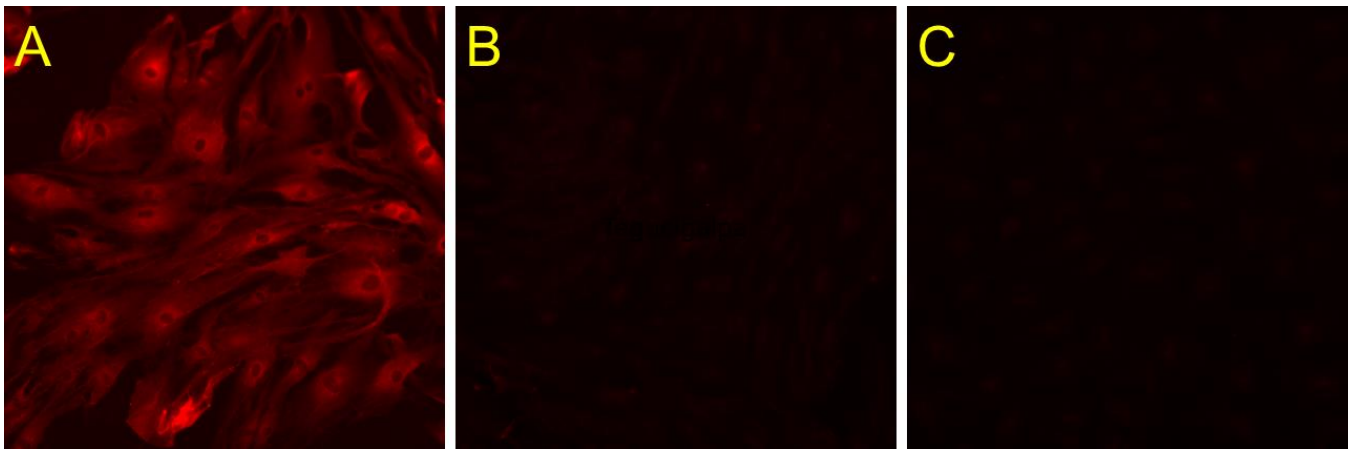

Image 1: Example images of myocilin expression after staining with MYOC antibodies. Three groups are shown, left to right: TM control (A), 4X Exosomes (B), and MSC control (C).

Group	Mean % of Well Area	p-Value (Against MSC samples)
TM	8.765% (n=5)	0.469
MSC	7.412% (n=6)	---
CMS	4.051% (n=2)	0.169
ExoCMS	8.959% (n=3)	0.468
4X	6.876% (n=4)	0.314
2X	5.757% (n=4)	0.250
1X	5.144% (n=1)	Sample Size too low

Table 2: Percentage of well after 12 hours in contractility gel, as well as p-values from heteroscedastic t-test against MSC samples. No group's change in area was significantly different than MSC group's response.

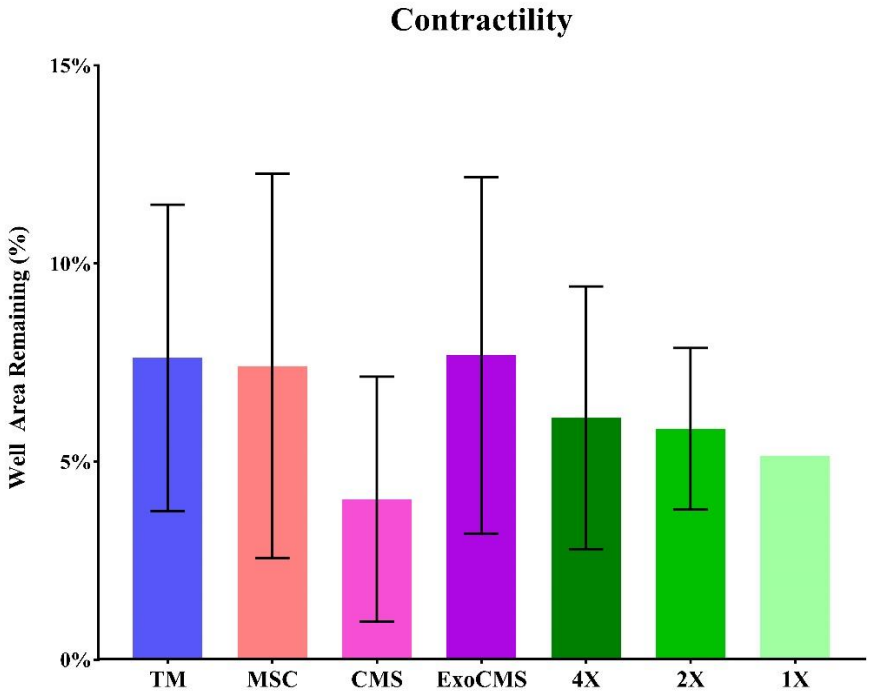

Figure 4: Area of contractile gels after 12 hours, as a percentage of 96-well area. Inclusion of EVs in experimental cells trends with less contraction. No groups statistically different from any other.

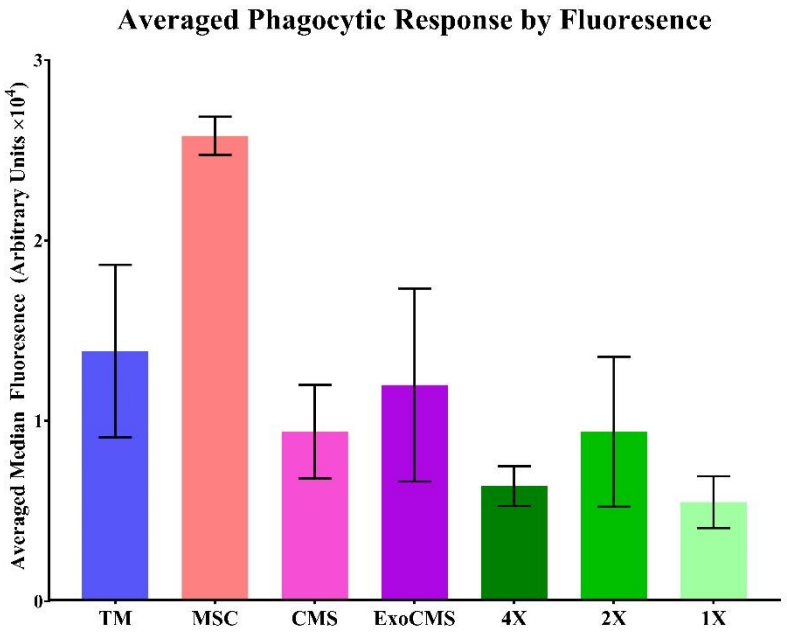

Figure 5: Mean fluorescence in samples given phagocytic particles normalized from cells not given phagocytic particle. Cells not given phagocytic particles had very similar fluorescence values (±110). Experimental groups tracked closely to TM control response, though variability in all samples prevented statements of statistical significance.

	TM	CMS	ExoCMS	4X	2X	1X
CHI3L1	683.0 ± 1173.9	123.5 ± 178.9	95.3 ± 26.4	11.5 ± 8.3	13.1 ± 14.5	53.5 ± 89.1
MGP	46.8 ± 80.8	3.4 ± 5.7	3.9 ± 3.4	4.4 ± 4.6	4.5 ± 7.7	26.8 ± 46.3
tPA	1.0 ± 1.0	2.9 ± 4.8	1.8 ± 1.3	2.7 ± 2.9	1.4 ± 1.7	1.0 ± 1.6
MYOC	71.1 ± 116.4	12.1 ± 11.1	8.8 ± 8.6	5.4 ± 5.0	4.5 ± 7.6	0.1 ± 0.1
MUC 1	1.2 ± 1.7	7.0 ± 12.0	8.3 ± 7.3	0.8 ± 0.7	1.8 ± 3.1	0.0 ± 0.1

Table 3: Fold change in expression, relative to MSC samples. All samples normalized against housekeeping genes TATA Binding Protein, YWHAZ, and GUSB.

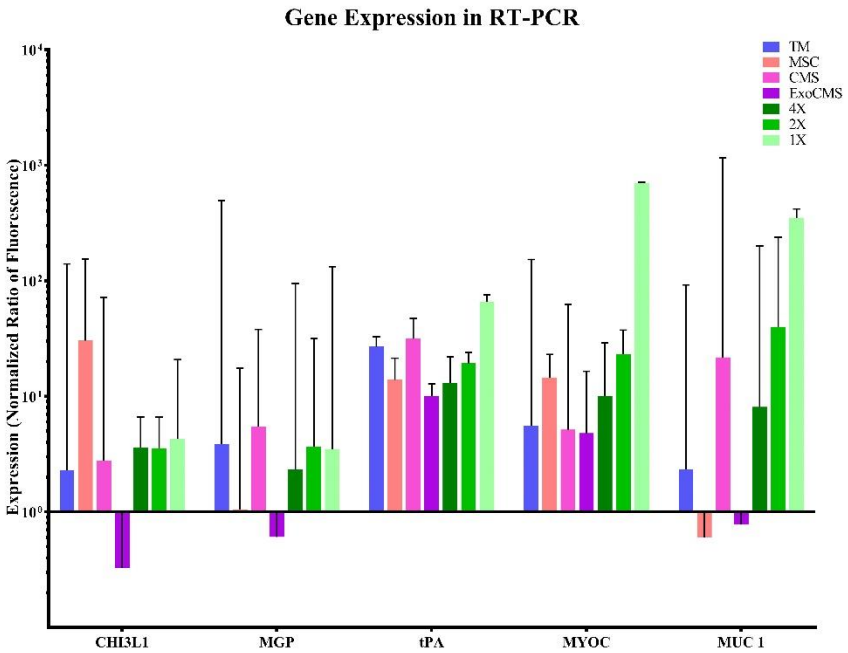

Figure 6: Fold-change in expression of TM and MSC marker genes in each experimental group. Most groups follow TM cell trends. Specifically, cells treated with EV's and media (ExoCMS group) tracked TM myocilin expression well, indicating trends in behavior.

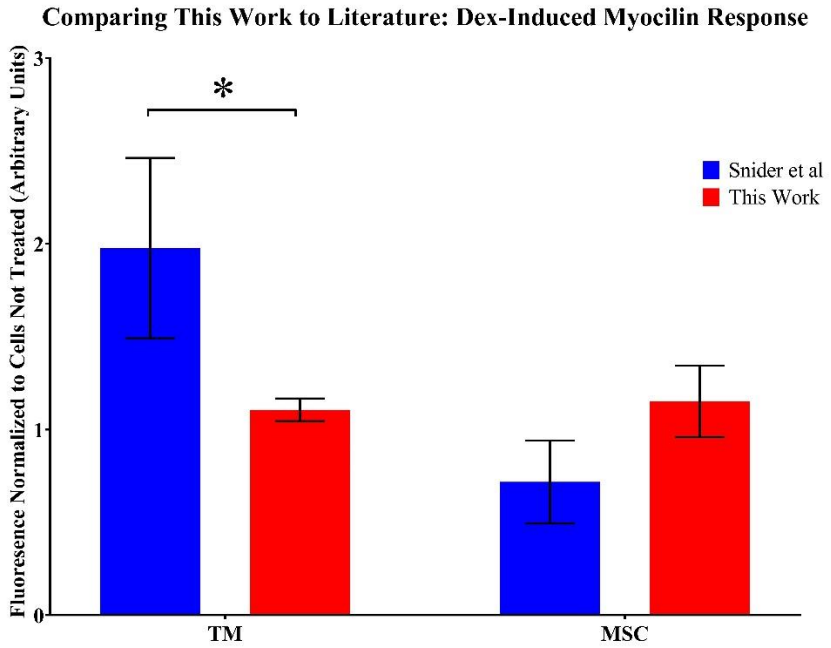

Figure 7: Dexamethasone-induced myocilin expression, as assessed by fluorescent antibodies, in this work's control TM and MSC cells and that of Snider et al. Expression in the two MSC groups did not significantly differ (p=0.3826), while TM cells were shown to be significantly different (p=0.0462).

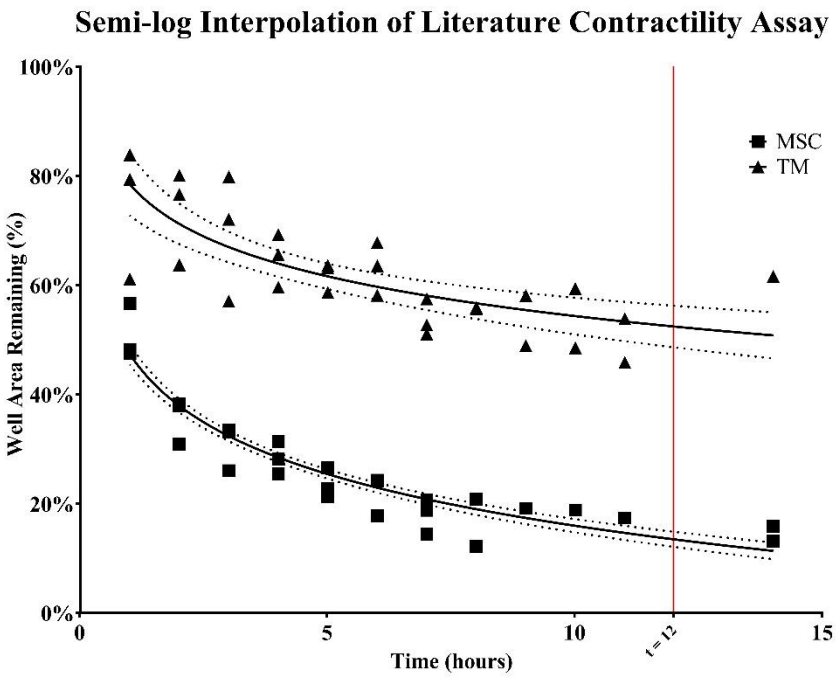

Figure 8: Interpolation of results from Snider et al. Percentage of well area at 12 hours was interpolated with 95% confidence for later comparison to this work.

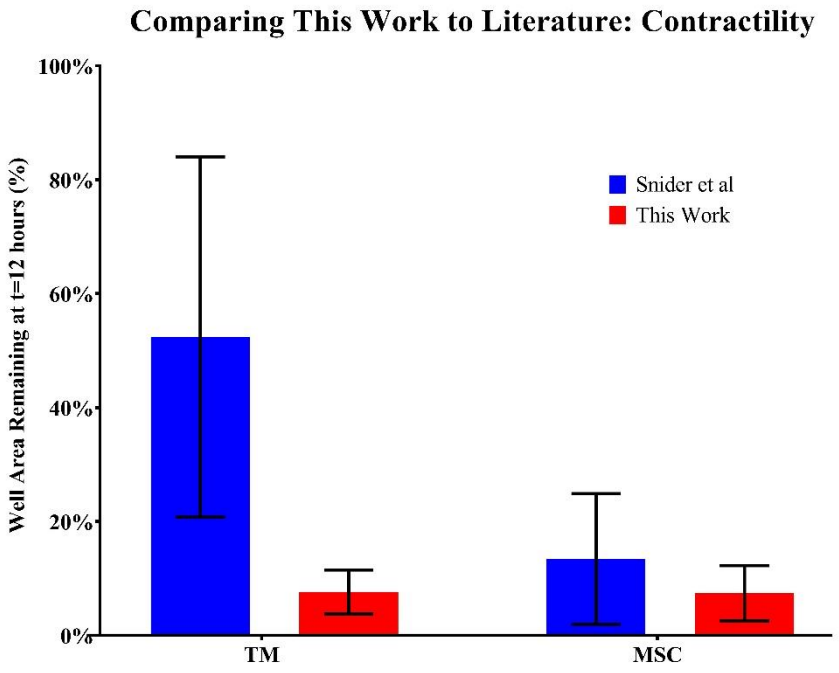

Figure 6: Comparison of contractile response, as percentage of well area after 12 hours, in this work's control TM and MSC and past literature. Past work's 12 hour response was interpolated with 95% Confidence. This work's MSC control's response could not be shown significantly different.

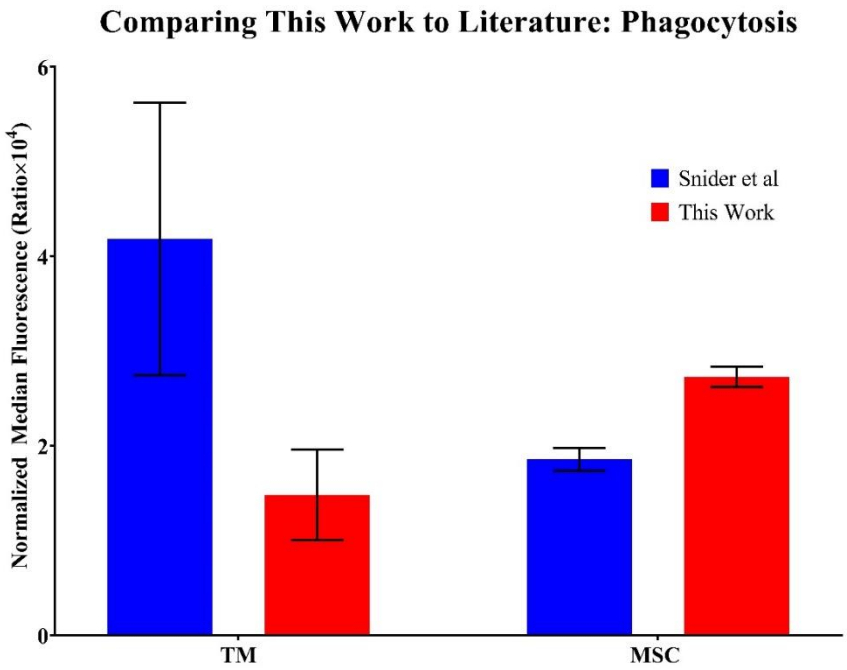

Figure 3: Phagocytic activity, as assessed by Fluorescently tagged E. coli, of two-week cultured cells against fresh, taken from Snider et al.'s work. TM cells differed much more than MSC's, indicating a susceptibility to loss in characteristics.

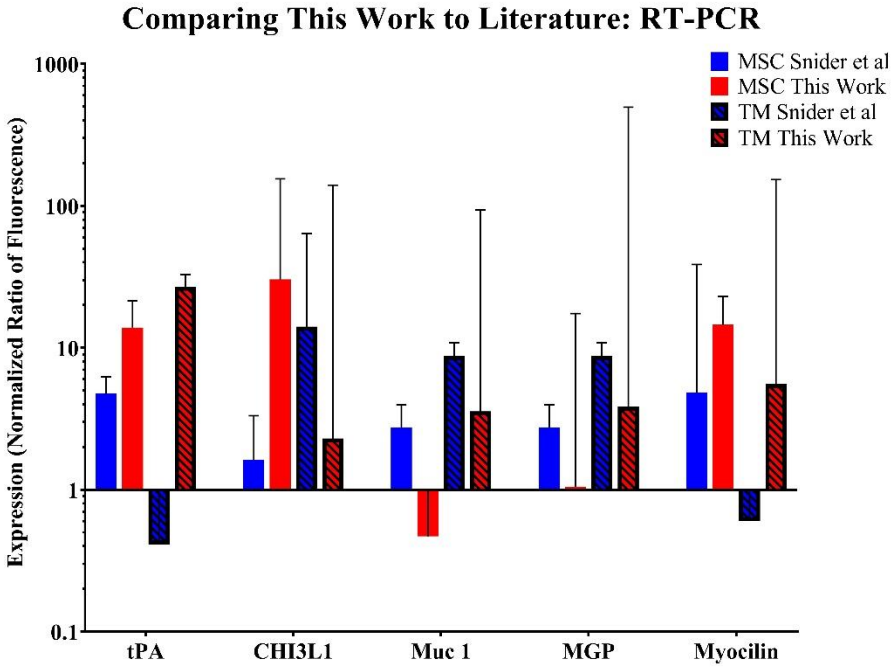

Figure 4: Comparison of TM and MSC RT_PCR cycles, normalized to housekeeping genes, from this work, after culturing through experiment, and past literature. No expression for both cell types were significantly different from each other.

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